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(54) Title: LABELING OF NUCLEIC ACIDS WITH FLUORESCENT MARKERS

(57) Abstract

The present invention relates to DNA markers and, particularly, nucleic acid labeling techniques. More specifically, this invention contemplates a protocol which permits the covalent introduction of single or multiple fluorescent markers or other probes into DNA fragments and oligodeoxynucleotides. The instant technique, particularly employing multiple fluorescent markers, allows high sensitivity detection of nucleic acids (without the use of sophisticated detection devices) in the low femtomolar (10^{-15} mol) range and additionally permits the placement of markers and probes at specific locations within the macromolecule. The present invention can be used with high detection sensitivity for DNA sequencing and hybridization procedures including a host of diagnostic and therapeutic procedures. The present technique can be employed as a tool for the study of nucleic acid dynamics through recognition and evaluation of fluorescence energy transfer and electron spin resonance, and the study of structure, conformation and dynamics of biopolymers. Specific labeling procedures allow the introduction of a probe or other entity for the location of desired sequences or the delivery of the probe to a specific sequence. This process is fundamental to the emerging fields of DNA diagnostics and therapeutics.

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LABELING OF NUCLEIC ACIDS WITH FLUORESCENT MARKERS

FIELD OF THE INVENTION

The present invention relates to DNA markers and, particularly, nucleic acid labeling techniques. More specifically, this invention contemplates a protocol which permits the covalent introduction of single or multiple fluorescent markers or other probes such as spin labels and drug analogues into DNA fragments and oligodeoxynucleotides. The instant technique, particularly employing multiple fluorescent markers, allows high sensitivity detection of nucleic acids (without the use of sophisticated detection devices) in the low femtomolar (10^{-15} moles) range and additionally permits the placement of markers and probes at specific locations within the macromolecule. The present invention can be used with high detection sensitivity for DNA sequencing and hybridization procedures including a host of diagnostic and therapeutic procedures. The present technique can also be employed as a tool for the study of nucleic acid dynamics through recognition and evaluation of fluorescence energy transfer and electron spin resonance, and the study of structure, conformation and dynamics of biopolymers. Specific labeling procedures allow the introduction of a probe or other entity for the location of desired sequences or the delivery of the probe to a specific sequence. This process is fundamental to the emerging fields of DNA diagnostics and therapeutics.

BACKGROUND OF THE INVENTION

The determination of the presence of nucleic acid fragments has typically relied on the use of radioisotopic labeling techniques. The enormous utility of these techniques has largely been a function of the high sensitivity associated with their detection. Such

sensitivity has allowed the location of quantities of material in amounts in the low femtomolar range (10^{-15} moles). However, the use of radioisotopes is rendered less than ideal by the associated problems of safety and disposal.

Fluorescent rather than radioisotopic labeling procedures are an attractive option which avoids these liabilities, but fluorescent labeling procedures have previously been compromised by their greatly reduced sensitivity. Fluorescent dyes as well as spin labels are also useful in many aspects of biophysics since the properties of a given marker can vary substantially with changes in the immediate microenvironment. Such probes can be useful for the study of structure, conformation and dynamics in biopolymers providing that they can easily be placed at specific locations within the desired macromolecule.

In order for fluorescent labeling procedures to compete effectively with and replace radioisotopic labeling techniques for the detection of macromolecules during various biochemical assays, the fluorescent labeling must result in high detection sensitivity, rapid and simple procedures for the introduction of the fluorescent marker to the macromolecule of interest must be available, and the results must be reproducible. By meeting these criteria and with the additional advantage of reduced health hazards, fluorescent labeling techniques could then replace the use of radioisotopes in a number of biochemical assays.

Intercalative dyes such as ethidium bromide generally meet these criteria and in many cases have completely replaced radioisotopic labeling procedures for the detection of double stranded DNA. However, a number of assays, including DNA sequencing and hybridization

techniques, cannot benefit from intercalative fluorescent labeling. These procedures require that the fluorescent marker be covalently bound to the nucleic acid, and the intercalative dye is unable to meet this requirement.

All prior studies for the covalent attachment of fluorescent markers to nucleic acids, until the present invention, suffered from at least one of two disadvantages. First, attachment of only a single label to the nucleic acid (usually at one of the termini) severely compromised its detection. Secondly, although multiple labeling techniques can enhance detection sensitivity, they have generally required the time-consuming synthesis of a modified nucleoside derivative containing a fluorophore or one which can be modified with a fluorophore. In addition to fluorophores, the use of biotin as a non-radioactive labeling technique has also been considered.

The use of single labels, usually at the terminus of the nucleic acid fragment, is the conventional state of the art primarily because it is chemically and enzymatically easier to exploit modification reactions at a nucleic acid terminus rather than at a specific point in the internal regions of the sequence. Additionally, the placement of the marker at one of these termini also removes the marker from the "site of action" when monitoring protein binding or any process where an essentially native DNA sequence is required. It has commonly been difficult to detect fragments containing a single fluorescent marker with the high sensitivity available with a radioisotopic label. Although problematic, labeling with a single fluorophore has been accomplished using both chemical and enzymatic techniques. DNA sequencing has been attempted using such labeling techniques but requires sophisticated electronic detection, and then only has evidenced limited success.

Several methods have been reported for the incorporation of multiple labels into nucleic acids. Most of these rely on an enzymatic polymerization reaction in order to introduce a modified nucleoside carrying the desired label or one which can be easily modified with the fluorescent marker at numerous positions. Base-specific reactions have also been employed, such as modification of guanine residues with N-acetoxy-2-acetylaminofluorene followed by detection with tetramethylrhodamine-labeled antibodies raised against the modifying reagent. Multiple labeling techniques have commonly resulted in enhanced detection sensitivity with respect to single labels and have been reasonably reproducible. However, these techniques have previously not been simple or rapid to employ. The modified nucleoside has previously only been obtained by time-consuming chemical syntheses.

Another prior approach involves the use of biotin labeling. While biotin itself is not a fluorescent chromophore, biotin labeling when combined with immunochemical, histochemical or affinity detection systems provides another alternative to radioisotopic labeling of nucleic acids. Biotin-labeled nucleic acids have been used in hybridization studies, gene mapping studies employing electron microscopy and gene enrichment in cesium chloride gradients. Biotin labeling has been typically approached in conceptually the same manner as fluorescent labeling techniques in which either a single label at the nucleic acid terminus or multiple labels requiring the synthesis of a biotin labeled dNTP derivative are employed. Generally, each of the existing techniques suffers from the requirements of arduous chemical synthesis and/or limited detectability.

Conventional techniques when applied to DNA sequencing procedures add additional complications since the DNA fragments prepared during sequencing techniques must be resolved by electrophoresis in a polyacrylamide gel matrix. Since electrophoresis procedures resolve nucleic acid fragments on the basis of size (or molecular weight), the addition of one or more fluorescent labels to the fragments prior to electrophoresis results in anomalous migration of the DNA within the gel and undue complications in the analysis of the sequence. The most desirable procedure for employing fluorescent labeling techniques in DNA sequencing and hybridization procedures would involve the incorporation of multiple labels into the nucleic acid or hybridization probe (to enhance detection sensitivity), before or after electrophoretic resolution of such fragments or before or after hybridization of the probe onto a nitrocellulose membrane ("pre-assay" or "post-assay" labeling). Multiple covalent labeling of nucleic acids with fluorophores in a "post-assay" manner has not been previously contemplated or described.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide an improved method for labeling nucleic acids.

Another object of this invention is to provide an improved method of fluorescently labeling nucleic acids.

A further object of the present invention is to provide new probes for use in DNA labeling and related techniques.

A still further object of this invention is to provide a new detection product which constitutes a phosphorothiolate diester covalently complexed with a nucleotidic residue, and which is also complexed with a detectable marker.

Another object of this invention is to provide multiple sites, i.e., internally within the macromolecule, for the attachment of fluorophores and other markers and/or probes to the nucleic acid thereby enabling multiple labeling techniques.

A further object of the present invention is to selectively introduce fluorescent markers and other markers and probes at specifically desired sites of the macromolecule. These markers or reporter groups include fluorophores, biotin, spin labels, drugs or their analogues, hydrolytic reagents, chiral metal complexes and the like.

Another object of this invention is to selectively introduce fluorescent markers and other probes after the molecule of interest has been treated with any one of various desired biochemical assays, i.e., in a "post-assay" procedure.

Still another object of this invention is to selectively introduce fluorescent markers and other probes before the molecule of interest has been treated with any one of various desired biochemical assays, i.e., in a "pre-assay" procedure.

Yet another object of the present invention is to provide an improved process for DNA sequencing, DNA hybridization techniques and DNA diagnostics and DNA therapeutics.

A still further other object of this invention is to provide a new detection procedure which eliminates the use of radioisotopes and the disadvantages associated with such conventional methods.

These and other objects of the present invention are achieved by providing a protocol which permits the covalent introduction of single or multiple markers, particularly fluorescent markers, and other probes into DNA fragments and oligodeoxynucleotides at selective sites. More

specifically, according to the present invention, nucleic acids are labeled with markers such that, e.g., the fluorescent marker or any other type of probe can be placed into a specific location in the nucleic acid. By the technique of the present invention, various sites for the attachment of the desired probes or markers are generated by employing phosphorothioate diesters in place of native phosphodiesters which are chemically or enzymatically introduced at the desired site within a nucleic acid and subsequently marked with the desired reporter group. The present methodology not only permits multiple labeling and high sensitivity in a simple technique in the absence of sophisticated detection devices, but also permits the introduction of a particular probe or marker after conventional biochemical assays, i.e., "post-assay." The advantages of the novel detection products of this invention also allow the labeling of DNA fragments in conventional DNA sequencing or hybridization assays. Such assays further permit a host of therapeutic procedures where a DNA hybridization probe with attached phosphorothioate diester(s) is employed in vivo or in vitro to locate a sequence within genomic DNA and which is subsequently reacted with, e.g., a label for detection or identification, a reactive molecule for degradation, or other toxic therapeutic agents. The novel product also allows study of the structure and dynamics of nucleic acids as well as protein-nucleic acid complexes. The novel product of the present invention includes a nucleotidic residue covalently complexed with a phosphorothioate diester and further complexed to a marker enabling detection of the product.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 sets forth the structure of the phosphorothioate triester composed of the nucleotidic residue and phosphorothioate diester complexed with the bimane label (bimane-Tp(S)T triester).

Fig. 2 is a graphic illustration of the stability of the bimane-Tp(S)T triester at ambient temperature measured during a total time period of 20 hours at pH values between 3-11.

Fig. 3 is a graphic depiction of an HPLC analysis of the reaction mixture containing the octamer d[GC(s)CCGGGC] (0.3 mM) and monobromobimane (3.0 mM) after reaction for 5 hours at ambient temperature.

Fig. 4 is a photographic reproduction of a polyacrylamide gel (6%) illustrating "post-assay" labeling of DNA fragments with monobromobimane.

Fig. 4(A) represents an HpaII restriction endonuclease digest of an M13mp18 DNA template, which has been elongated with DNA polymerase I (E. coli) using dNTPs and then treated with the endonuclease.

Fig. 4(B) represents an AvaI restriction endonuclease digest of an M13mp19 DNA template, which was elongated with DNA polymerase I (E. coli) using dNTPs and then treated with endonuclease.

Fig. 5 represents phosphorothioate triester oligodeoxynucleotides carrying (a) a PROXYL spin label. (b) a derivative of the dihydropyrroloindole subunit of CC-1065, (c) a sulfonamide-linked dansyl fluorophore, and (d) an N-linked dansyl fluorophore.

DETAILED DESCRIPTION OF THE INVENTION

The present invention contemplates the selective labeling of nucleic acids with fluorescent molecules and other probes such as, for example, biotin, which are useful in DNA sequencing and DNA hybridization assays. The present

invention also contemplates other probes such as, for example, spin labels which are useful in the analysis of nucleic acid structure and dynamics. The convenient labeling methodology of this invention further permits a broad range of DNA therapeutic and diagnostic procedures and is particularly characterized by the selective covalent introduction of single or multiple markers and probes into DNA fragments and oligodeoxynucleotides. The novel detection product of this invention is characterized by a nucleotidic residue covalently complexed with a phosphorothioate diester which is mutually covalently complexed with a selected marker. The probe is selectively introduced into a single site of choice or into multiple sites as desired.

The present invention preferably employs a phosphorothioate diester [for example, Tp(s)T, phosphorothioate diester derivative of TpT (thymidyl(3'→5') thymidine)] which is selectively incorporated into a DNA fragment or oligodeoxynucleotide at any and each nucleotide residue desired.

Specifically, the probe of the present invention, a phosphorothioate diester derivative, is prepared by introducing the phosphorothioate diester into the nucleic acid fragment either enzymatically, e.g., according to the method of Potter and Eckstein (Potter, B. and Eckstein, F., J. Biol. Chem., 259: 14243-14248, 1984), or chemically, e.g., according to the method of Connolly, et al. (Connolly, et al., Biochemistry, 23: 3443-3453, 1982).

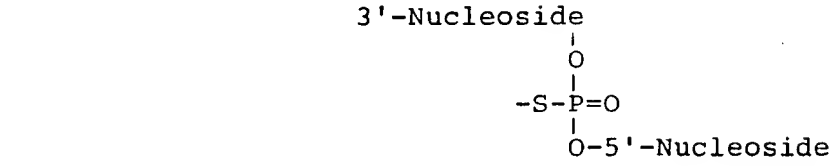
The enzymatic technique of Potter and Eckstein employs the desired dNTPαS 2'-deoxynucleoside-5'-0-(1-thiotriphosphate), a suitable enzyme with polymerizing characteristics such as DNA polymerase or reverse transcriptase, a DNA template and a primer. The enzyme employed, uses dNTP S as a substrate to synthesize nucleic acids of varying chain length, and upon enzymatic reaction, a

phosphorothioate diester is incorporated between two nucleoside residues, along with the concurrent liberation of pyrophosphate.

The phosphorothioate diester may be introduced chemically into the nucleic acid by the method of Connolly, et al. (or Stec, et al., J. Am. Chem. Soc., 106: 6077-6079). This is generally a three step procedure. First, a phosphite triester (nucleoside phosphite triester) is formed by reacting a nucleoside phosphoramidite in the presence of a weak acid such as tetrazole. Second, the phosphite triester is oxidized in the presence of elemental sulfur (S_8), CS_2 and lutidine, to form a phosphorothioate triester complex. Third, in the presence of a base such as ammonia, the phosphorothioate triester is hydrolyzed to the desired phosphorothioate diester.

The selective introduction of the phosphorothioate diester derivative into the DNA fragment or oligodeoxynucleotide, is determined by the choice of oxidation procedures at any given position. As explained above, the phosphorothioate diester is obtained by oxidation in the presence of S_8, CS_2 and lutidine. The native phosphate diester is obtained by oxidation of the phosphite triester with a mixture of I_2, THF (tetrahydrofuran), H_2O and lutidine followed by hydrolysis of the triester to yield a phosphate diester. The appropriate choice of either set of conditions allows the placement of the phosphorothioate diester in the desired position with respect to the native phosphate diester. This technique allows for selective reactivity at a specific nucleotidyl site, and avoids nonspecific reaction with other functional groups available in the nucleic acid.

The complex formed is described below:



(Internucleotidic Phosphorothioate Diester)

The phosphorothioate diester can subsequently be alkylated with fluorescent molecules or other probes such as, for example, biotin. In this procedure, the complex which results is referred to as a "phosphorothioate triester" (which comprises an internucleotidic residue, a phosphorothioate diester and a detectable marker). The means by which this procedure occurs, e.g., alkylation, refers to the displacement of the functional group (such as the bromine in monobromobimane) and the formation of a sulfur-carbon bond between the fluorescent marker and the phosphorothioate diester.

For purposes of fluorescent labeling techniques herein contemplated, various fluorophores can be employed, for example, monobromobimane (MBB), bromomethylcoumarin, as well as a variety of chromophores carrying bromoacetamides, iodoacetamides, aziridinosulfonamides or γ-bromo-α,β-unsaturated carbonyls; monobromobimane is preferred.

One of the most surprising advantages of this invention is that the present methodology permits the introduction of fluorescent dyes or other probes in a "post-assay" procedure. By "post-assay" procedure is meant, generally, that the phosphorothioate diester-containing DNA is used in the assay of choice, for example, in polyacrylamide gel electrophoresis, and the fluorescent molecule or other marker or probe can be introduced at a later time, for example, while the nucleic acid is embedded

in the polyacrylamide gel matrix. The assay procedures contemplated by the present invention in this context include, for example, gel electrophoresis, Southern hybridization, and DNA sequencing techniques such as are described by Sanger, et al. (Sanger, et al., Proc. Natl. Acad. Sci., 74: 5436-5467, 1977).

Gel electrophoresis as used here is typically performed by running DNA samples down specific lanes in a gel (e.g., a polyacrylamide gel or agarose gel), under controlled current and temperature conditions for a short period of time. This procedure leaves the DNA embedded in the gel matrix.

Southern hybridization involves the use of a blotting membrane to remove the fractionated nucleic acid from the gel and allows for hybridization of labeled probes to the nucleic acid on the surface of the blotting membrane. Radioisotopic labeling (^{32}P) has been commonly employed for the detection of nucleic acids resolved by electrophoresis or after hybridization techniques.

Sanger DNA sequencing (also known as "dideoxy sequencing") has previously been done using ^{35}S labeling. This typically involves two steps. The labeling reaction is initiated after annealing of the primer to the template. A low concentration of dTTP, dGTP, dCTP and α-[^{35}S]dATP is employed in order to elongate the primer and incorporate some radioisotope. The second step involves adding the termination mixture, which is a higher concentration of all four dNTP derivatives plus one of the dideoxy derivatives (ddNTP).

Post-assay fluorescent labeling techniques as described herein permit the introduction of multiple fluorescent molecules or other appropriate markers into the

nucleic acid, e.g., after electrophoresis and "post-assay" labeling of detecting oligodeoxynucleotides and DNA fragments can be detected on the basis of, e.g., fluorescence, with high sensitivity.

Detection of fluorescent markers can be achieved by use of e.g., a standard long-wavelength ultraviolet transilluminator, to view the DNA in the gel.

The labeling procedure is particularly useful in conventional enzymatic procedures for the sequencing of DNA. Instead of radioisotopic labeling as described in the Sanger sequencing technique the four dNTPαS derivatives used in the sequencing reaction can be substituted such that the DNA fragments produced will contain phosphorothioate diesters at all internucleotidic positions which can allow multiple labeling and ultimately allow reading of large and small DNA fragments. The labeling procedure is also applicable to site specific identification of nucleotides by introducing at least one phosphorothioate diester selectively into an internucleotidic residue or DNA fragment or oligodeoxy-nucleotide, labeling said phosphorothioate diester with a marker and detecting said marker.

The aforesdescribed labeling technique can also be applicable to hybridization studies using, e.g., membrane-bound nucleic acids.

A fluorescently labeled cloned DNA probe can be used to localize specific nucleic acid sequences in mixtures of DNA restriction fragments fractionated by gel electrophoresis. A replica of the gel is made by transferring all of the fractionated DNA fragments to a sheet of nitrocellulose paper or similar membrane (the "blotting membrane") by diffusion or electrophoresis. The hybridization probe can be labeled before or after the

hybridization assay occurs. The locations of the fragments that hybridize to fluorescently labeled DNA probes are then identified by their fluorescence. Similarly, nitrocellulose paper replicas can be made of crowded colonies of bacteria growing on an agar surface so that hybridization of the paper with a specific labeled probe can be used to identify the few cells carrying a newly cloned specific DNA fragment.

The labeling and detection techniques herein discussed, can also surprisingly be easily employed in DNA diagnostics and DNA therapy. The present advantage, relative to art recognized techniques, is particularly manifest in that the presence of the phosphorothioate diester does not effectively alter the biophysical nature of the DNA and yet selectively introduces a nucleophilic site which is readily modified and exploited for diagnostic and therapeutic purposes. For example, the phosphorothioate diester can be introduced into the DNA and subsequently hybridized to a gene of interest in vitro or in vivo, and then followed by specific introduction of a probe to that gene. The probe to the particular gene can then be used to discover the location of the gene. This leads to detection of the presence or absence of the gene under diagnostic investigation. The probe can then be used in DNA therapeutics to inactivate or destroy that particular gene or if necessary, to activate that gene. For example, diagnosing genetic disorders and direction of drug delivery (e.g., anticancer or antiviral drugs).

Another surprising advantage of the present invention is that the DNA-containing phosphorothioate diester is largely resistant to nucleases and therefore is very stable when introduced into complex biological systems found in vitro and in vivo.

The present invention can be used in spectroscopic analysis (e.g., Nuclear Magnetic Resonance studies, and in particular, the Nuclear Overhauser Enhancement [NOE]) to measure distances within nucleic acids by use of probes which can label specific phosphorothioate diesters.

The present invention can also be applied to Electron Spin Resonance studies, which previously relied upon the use of non-specific labeling. The simple and rapid procedures described here will allow the preparation and study of nucleic acid fragments containing spin labels, attached at well-characterized locations. The proceudre described herein can also be used for the specific attachment of hydrolytic reagents (e.g., ferric ion complexes), intercalators and proteins to nucleic acids.

Additionally, the present invention can also be used to probe the structure of DNA fragments or oligodeoxynucleotides by using chiral metal complexes (e.g., the Λ-isomer or Δ-isomer of tris-(4,7-diphenylphenanthroline) cobalt (III)) as the one marker of choice to be attached to the phosphorothioate diester.

In order to use the phosphorothioate diester effectively in a procedure for detecting nucleic acids, it is advantageous to assess the stability, particularly with respect to pH, of the labeled phosphorothioate diester-fluorescent marker product. An HPLC analysis can be used employing a reversed phase column. This assays the stability of the labeled phosphorothioate derivative (triester) over a broad pH range during an incubation period at ambient temperature.

In another aspect of the present invention, high detection sensitivity of fluorescent labeled nucleic acids can be facilitated by the introduction of multiple

fluorescent markers to a corresponding multiple number of phosphorothioate diesters earlier introduced at the selected internucleotidic sites; the labeling reaction must occur at adjacent phosphorothioate diesters such that, to achieve maximum sensitivity, a nucleic acid fragment carries a fluorophore at each and every internucleotidic phosphorus residue. Surprisingly, experimentation indicates that there is no steric hindrance or other difficulty in placing fluorescent labels on adjacent phosphorothioate diesters, thus permitting maximization of this technique.

As earlier discussed, "post-assay" labeling procedures are useful for a variety of biochemical assays; one of the most important specific applications involves the detection of nucleic acids resolved by gel electrophosesis techniques. One "post-assay" labeling procedure, for example, can be accomplished using short oligodeoxynucleotide fragments resolved by a given assay (e.g., gel electrophoresis) and then soaking the gel containing the small nucleic acid fragment with a solution which contains the fluorescent marker of choice. Small fragments with several labeled phosphorothioate diesters are quantitatively compared with the fluorescence exhibited by a nucleic acid fragment with a single fluorophore. There is a concomitant increase in detection sensitivity with an increase in the number of labeled phosphorus residues.

Longer DNA fragments containing phosphorothioate diesters can be prepared by enzymatic synthesis when the normal dNTP substrates are replaced by α-thio derivatives (dNTPαS). In order to generate fragments of defined length, an oligodeoxynucleotide primer can be extended using a template (e.g., M13mp18 or M13mp19 or other single-stranded DNA) and then the resulting material can be hydrolyzed with

an appropriate restriction endonuclease. The amount of DNA fragment which can be visualized is approximated based upon the maximum amount of template present in the reaction mixture or as the result of internal standardization via radioisotopic labeling. The variety of bands produced can be visualized by "post-assay" fluorescent labeling procedures. The results show a further increase in sensitivity relative to the increased sensitivity in small nucleic acid fragments.

Various fluorophores are available and many can be employed in the present process. Any fluorophore can be utilized for the "post-assay" fluorescent labeling procedures contemplated by the present invention which reasonably possess the following properties: high quantum yield; solubility in aqueous (or largely aqueous) solutions; relatively small size to allow diffusion through the gel matrix; high fluorescence only after reaction with a sulfur residue; and removal of the excitation maximum from the absorbance maximum of the nucleic acids. One preferred fluorophore which meets these criteria is monobromobimane. Other fluorophores of choice can include, for example, bromomethylcoumarin, or fluorophores carrying bromo- or iodoacetamides, or aziridinosulfonamides. The fluorophores of choice have the ability to alkylate the phosphorothioate diester. The phosphorothioate diester is more nucleophilic than any other site on the nucleic acid and results in formation of a stable phosphorothioate triester when labeled with the fluorophore of choice.

In particular, two widespread assays which can be employed in conjunction with the "post-assay" fluorescent labeling of this invention are DNA sequencing using, e.g., the Sanger dideoxy method and DNA hybridization (using e.g., the Southern technique).

1. DNA Sequencing

Post-assay labeling is most amenable to enzymatic dideoxy sequencing procedures. This approach incorporates phosphorothioate diesters in place of native phosphate diesters in the DNA fragments generated. After gel electrophoresis, multiple fluorophores, such as MBB, can be attached to the DNA via alkylation of the sulfur residue of the phosphorothioate diesters.

Current technology of Sanger sequencing utilizes the dNTP derivatives. The Sanger sequencing technique commonly utilizes a single α-[S^{35}]dNTP derivative to introduce the readioactive label. However, by using all four dNTPαS derivatives in the present invention, DNA fragments can be generated by this technique which can contain hundreds of phosphorothioate diesters. The "post-assay" labeling of this invention can be directly applied to the detection of these fragments.

The "post-assay" fluorescent labeling technique provides the sensitivity necessary to visualize DNA sequencing ladders in the absence of radioisotopes. The technique as described here employs all four dNTPαS derivatives plus one of the dideoxy derivatives (ddNTP) in the elongation and then termination of the DNA primer. Sequencing ladders can be generated with dNTPαS substrates in the like manner to the methodology with dNTP derivatives.

It is then desirable to vary the elongation and termination conditions such that in the initial fluorescence labeling the amount of DNA in each band may be varied. Then the amount of DNA that appears in the bands can be maximized, e.g., ranging from approximately 300 to 500 base pairs. Fragments of this size can be resolved, and 300 to 500 fluorophores or other types of markers can be incorporated

into such fragments. The distribution of the fragments can be altered by changing the relative ratios of the dideoxynucleotide/deoxynucleotides triphosphates.

A ddNTP/dNTP∝S ratio of about 1:10 may be used to obtain a distribution of small and large fragments. A decrease in this ratio is effected to allow for more efficient polymerization in a stepwise manner to as low as about 1:500 in order to shift the distribution to longer fragments.

The use of ∝-[^{35}S]dATP as a method for introducing the radioisotopic label has been reported and is commonly employed. Dideoxy sequencing using ^{35}S labeling typically involves two steps. After annealing of the primer to the template the labeling reaction is initiated. A low concentration of dTTP, dGTP, dCTP and ∝-[^{35}S]dATP is employed in order to elongate the primer and incorporate some radioisotope. The second step involves adding the termination mixture which is a higher concentration of all four dNTP derivates plus one of the dideoxy derivatives (ddNTP). It is a simple procedure to then substitute the four dNTP∝S derivatives in both reactions (actually there is only one reaction since no radioisotopic labeling is involved) such that the DNA fragments produced will contain phosphorothioate diesters at all internucleotidic positions.

For internal standardization, radioisotopic labeling can be used in combination with fluorescent markers to monitor the limits of detection sensitivity. To obtain fragments which have been labeled to a known specific activity a "minus-dCTP" labeling reaction is employed. This uses a primer and template of known sequence, for example, of the following sequences:

M13mp18 3'...CAAAAGGGTCAGTGCTGCAACATTTTGCT...5'
primer 5'-GTTTTCCCAGTCACGAC-3'

The labeling reaction can now be performed with low concentration of the dTTP∝S, dGTP∝S and ∝-[^{35}S]dATP. The elongation of the primer proceeds until the first dG present in the template and then terminates resulting in the following sequence containing four ^{35}S labels:

M13mp18 3'...CAAAAGGGTCAGTGCTGCAACATTTTGCT...5'
elongated primer 5'-GTTTTCCCAGTCACGACGTTCTAAAA-3'

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The termination reaction uses all four dNTP∝S derivatives at concentrations some two orders of magnitude higher than the labeling reaction such that any remaining radioactive ∝-[^{35}S]dATP is diluted and the quantity available for incorporation becomes insignificant. The amount of material present (based upon the known specific activity of the ∝-[^{35}S]dATP) in a given band can now be easily determined by excising the band, lyophilizing the gel and determining the radioactivity present by scintillation counting. By adjusting the concentrations of the template and primer as well as the ratio of the ddNTP to dNTP∝S, the amount of DNA present in a given fragment can be altered. In addition, distribution of fragments can be shifted to those of higher or lower molecular weight. Optimization of detection can allow "reading" of smaller fragments (smaller than 300 nucleotide residues). DNA sequencing in the abscence of radioisotopes can then be effectuated by detecting the hundreds of labeled, e.g., bimane-labeled phosphorothioate triesters by utilization of single or sophisticated electronic techniques.

2. DNA Hybridization

In another embodiment of the present invention, the post-assay fluorescent labeling technique can also be applied to hybridization studies using nucleic acids. The stability of a native DNA duplex is first tested against nucleic acid containing a number of phosphorothioate diesters and the effect of this stability when the phosphorothioate diesters are alkylated by a fluorophore is determined. For example, the results for the detection of a 21-mer fragment containing 20 phosphorothioate diesters shows that in the absence of electronic instrumentation it can readily be detected visually. Nucleic acids with one label can be detected and detection of single nucleotides can be facilitated. Such visibility is increased proportionatly with the proportionate number of markers.

A 21-mer fragment is one example of a small hybridization probe which can be used to detect nucleic acid sequences. This is utilized in the following manner: DNA fragments or oligodeoxynucleotides of reproducible size are generated by selective chemical means, such as by a restriction endonuclease enzyme. These nucleic acids are resolved by a biochemical assay such as polyacylamide or agarose gel electrophoresis. The nucleic acid resolved in this manner is then transferred to a blotting membrane, e.g., nitrocellulose membrane and the DNA probe is hybridized to the nucleic acid. Although the DNA probe at this point has the phosphorothioate diester or diesters incorporated into it, the marker of choice, e.g., a fluorescent marker, may be introduced before or after the hybridization assay. Following these steps, the marker can be detected using simple or sophisticated detection techniques.

One of the primary differences between "post-assay" fluorescent labeling within a gel matrix and labeling on a blotting membrane is that the latter occurs primarily on the surface of the membrane and not within a three dimensional matrix. With such surface phenomena it is possible to also use biotin labeled hybridization probes and detection with fluorescent protein complexes which could not be used for labels embedded in a gel matrix (the proteins involved are of large molecular weight and would not readily diffuse through the pores of the gel matrix). The phosphorothioate diester can be employed to allow efficient multiple (and specific) labeling with a biotin derivative. For example, the bromoacetamido group can be used to modify the phosphorothioate diester. A biotin derivative containing this functional group can be prepared quite simply by techniques available to one of ordinary skill in the art. Biotin labeling in this manner is considered an effective method for detecting nucleic acids when combined with immunochemical, histochemical or affinity detection systems. Two similar proteins, avidin and streptavidin, bind biotin very strongly and when coupled to fluorescent markers, enzymes or electron-dense proteins, can be exploited for the detection of nucleic acids. The use of fluorescent labeled antibodies raised against biotin can also be employed for detection. The biotin-labeled hybridization probe may be detected by use of a commercially available kit used for the detection of fluorescently labeled antibodies or by use of a transilluminator to detect the fluorescent group or protein.

Hybridization assays require the hybridization probe form stable Watson-Crick base pairs in order to localize the probe at a given sequence. The addition of biotin derivatives to the internucleotidic phosphorus

residues can result in some destabilization of the double stranded hybridization product. A series of biotin labeled probes can be prepared containing from one to approximately five biotin labels and the stability of the duplexes formed can be examined with biotin modified oligodeoxynucleotides in comparison with those unmodified. This can be accomplished by labeling of the oligodeoxynucleotides containing the correctly positioned (and number of) phosphorothioate diester(s) and isolation of the product using HPLC techniques. Duplex stability can be monitored by thermal denaturation experiments and circular dichroism spectra.

The ability of the biotin labeled oligodeoxynucleotide to function as a hybridization probe can then be examined using, for example, the 21-mer previously described. The sensitivity to detection of probes containing a varying number of biotin labels can be examined using commercially available fluorescent labeled proteins. "Spacing" the labels every two, three or more phosphorus residues can be the simplest route to enhance detection sensitivity.

In a second approach involving "post-assay" labeling, the phosphorothioate-containing probe is hybridized in one step; this avoids problems with the instability (if any) of the biotin labeled hybridization product. Subsequently, modification with the biotin label occurs, and after removal of the excess label, the protein solution is added for detection. This approach is conceptually similar to the one described for the visualization of DNA sequencing ladders and may also be the simplest approach to hybridization assays.

Hybridization experiments can also be performed with relatively long DNA fragments obtained from restriction digests and multiple phosphorothioate diesters can be

incorporated into such a fragment using DNA polymerase and nick-translation procedures. Radioisotopic labeling is accomplished by introducing "nicks" in the DNA with a dilute solution of DNase I and then elongating the nicked sites using DNA polymerase and the α-[^{32}P]dNTP substrates. The radioisotopic derivatives can then be replaced with the dNTP S derivatives and then hundreds of phosphorothioate diesters can be incorporated into the fragment. The simplest system to test hybridization can be one involving the M13 DNA being used in the sequencing reactions. For example, M13 RF (replicative form) DNA can be prepared in the conventional manner and then cleaved out a 444-mer to use as a hybridization probe. The 444-mer can then undergo nick-translation to incorporate the phosphorothioate diesters and then the modified and native sequences resolved by gel electrophoresis. A second sample of the M13 RF DNA, for example, can be digested such that the complementary 444-mer restriction fragment (in additon to others) is produced and transferred from an agarose gel to nitrocellulose or similar blotting membrane. The hybridization can then proceed followed by post-assay fluorescent labeling using, e.g., monobromobimane; fluorescent labeling with hundreds of markers provides the desired detection sensitivity. Since the monobromobimane is largely non-fluorescent until it alkylates a sulfur containing functionality, the membrane background fluorescence is relatively low. The labeled marker can then be detected with relative ease.

In another embodiment of the present invention, DNA probes are generated from mRNA. Again, one can simply use the dNTP S derivatives, which function as substrates for reverse transcriptase, to form the complementary DNA strand for use as a hybridization probe. The use of the new

labeling approach provides well-characterized hybridization probes which can be used for the detection of specific DNA sequences, in the absence of radioisotopes, for example, in Southern blots, Northern blots, colony screening or plaque screening.

3. Specific Modification of Nucleic Acids with Fluorescent Markers or Spin Labels

In a further aspect of this invention, the labeling of specific phosphorothioate diesters is also valuable for structural studies involving fluorescent energy transfer techniques and electron spin resonence (ESR) techniques.

The application of these two spectroscopic techniques has long suffered from the difficulty in specifically attaching the desired probe to the nucleic acid fragment. The present procedure permits simple and rapid synthesis of a variety of nucleic acid sequences which can be easily modified with fluorescent markers or spin labels for spectroscopic studies.

Fluorescent Energy Transfer Techniques allow for a simple and rapid means for measurement of longer distances within the nucleic acid structure, complementing NMR techniques such as that of the Nuclear Overhauser Enhancement (NOE) which can only measure small distances in the nucleic acid.

The disadvantages of the energy transfer technique have previously been in the difficulty of easily placing the donor and acceptor chromophores in specific positions, and the questionable accuracy of the technique when the orientation of the chromophores is unknown.

These two shortfalls are eliminated by the labeling of specific phosphorothioate diesters pursuant to the methodology of the present invention. By controlling the

position of the phosphorothioate diester, the placement of a specific label becomes as rapid as it is simple. Since the label is oriented on the outer surface of the macromolecule a freely rotating chromophore is likely.

ESR spectra can be valuable for the study of biopolymer dynamics providing that the appropriate spin label can be specifically bound to the macromolecule of interest. In general, the technique has suffered a similar disadvantage to energy transfer experiments in the difficulty of specifically placing the label on the macromolecule. The use of the phosphorothioate diester can again be valuable in this respect. Nucleic acid fragments can be prepared with spin labels by exactly the same approach as described above for fluorescent markers. Specifically labeled probes can be designed and prepared for these ESR studies.

Other procedures which can be used in association with the instant technique involve optimization of fluorescence detection. These include, for example, 1) altering the microenvironment of the labeled nucleic acid fragments in the gel matrix to increase the quantum yield of the fluorophore, 2) adjusting the excitation light energy to optimally fit the excitation spectrum of the dye and using filters to screen out all light energy (largely excitation wavelengths) other than the desired emission energy, and 3) examining electronic detection as a means of automating the reading of the information present. The first two approaches together can be expected to increase the detection sensitivity by roughly one order of magnitude. Electronic methods can be expected to provide one or more additional orders of magnitude enhancement.

The following examples would assist in further detailing the subject invention herein.

EXAMPLES

1) Chemical Oligodeoxynucleotide Synthesis

Tp(s)T, the phosphorothioate diester derivative of TpT, is an example of the simplest phosphorothioate diester amenable to the labeling procedures described.

The $(dT)_{15}$ with phosphorothioate diesters 3' to thymidine residues 7, 8, and 9 were synthesized by using the phosphite triester methodology (Beaucage & Caruthers, Tetrahedron Lett., 22: 1859-1863, 1981) on a solid-phase CPG support. The synthesis was interrupted prior to the oxidation step when the incorporation of a phosphorothioate diester was desired. The normal oxidation step with 0.1 M I_2 in tetrahydrofuran/distilled water/lutidine (40:1:10) was replaced with a solution of 2.5 M sulfur in CS_2/lutidine (1:1). The sulfur oxidation solution was injected directly onto the column with a syringe. After a reaction time of 1 h at ambient temperature, the column was washed with a 1:1 solution of CS, and lutidine to remove the residual sulfur. The column was then replaced on the machine, and the synthesis cycle was resumed. The 21-mer d(GCTATCGAAAGATCTCATAAG) was synthesized in an analogous manner. The synthesis was interrupted at every oxidation step to allow oxidation with the sulfur solution.

Both oligodeoxynucleotides were deprotected in ammonia at 50°C for 18 h. Isolation was done by reverse-phase HPLC on a 9.4 x 250 mm column of MOS-Hypersil using a buffer of 50mM triethylammonium acetate, pH 7.0 with a gradient of 20-65% acetonitrile in 40 min.

2) Solution Fluorescent Labeling Studies

The fluorophore of choice in this example, monobromobimane (MBB), was dissolved in acetonitrile, and stock solution (100mM) was stored in the dark at -20°C.

Typically, the oligodeoxynucleotides of interest were treated with an excess of monobromobimane, and the reaction was monitored by HPLC. Specifically, a solution of Tp(s)T (3.6 mM) in water was allowed to react overnight (18 h) with a 6-fold excess of monobromobimane (22 mM). The octamer (0.3 mM) in water was allowed to react with either a 5-fold excess of MBB (1.5 mM) or a 10-fold excess of MBB (3.0 mM). The fragment Tp(s)Tp(s)Tp(s)T (0.43 mM, a phosphorothioate diester concentration of 1.29 mM) was treated with an 8-fold excess (with respect to the phosphorothioate diesters) of MBB (10.5 mM). Covalent fluorescent labeling of the 15-mer in solution (0.8 mM) with MBB was achieved at 7.5 mM MBB (3-fold excess for 2.4 mM phosphorothioate diester).

The bimane-labeled Tp(s)T (see Figure 1) was isolated by reverse-phase HPLC on a 4.6 x 250 mm column of ODS-Hypersil with 50 mM triethylammonium acetate, pH 7.0, and a gradient of 0-70% acetonitrile in 1 h. The other labeling reactions were monitored by reverse-phase HPLC on a 4.5 x 250 mm column of ODS-Hypersil with either 20 mM KH_2PO_4, pH 5.5, and a gradient of 0-70% methanol in 30 min (the octamer and tetramer) or 50 mM triethylammonium acetate, pH 7.0, and a gradient of 0-35% acetonitrile in 1 h (15-mer).

Thin-layer chromatography studies were performed on silica gel thin-layer plates with a mobile phase of dichloromethane/methanol (9:1).

3) pH Stability Studies

Duplicate reaction mixtures of 6 nmol of bimane-labeled Tp(s)T were incubated at ambient temperature in 50 mM buffer at the appropriate pH values. The following buffers were used: pH values 3, 4 and 5, acetic acid/potassium acetate; pH values 6 and 7, K_2PO_4/K_2HPO_4; pH values 8 and 9, Tris-HCl; pH values 10 and 11, CAPS. At various reaction times, the samples were analyzed by HPLC on a 4.6 x 250 mm column of ODS-Hypersil using 0.02 M potassium phosphate, pH 5.5, with a linear gradient of 0-70% methanol in 30 min. The bimane-labeled Tp(s)T eluted at 21 min, while the product TpT eluted at 16 min.

At low pH values (3-7) less than 5% of the triester was hydrolyzed after a 20 h incubation as determined by integration of the corresponding HPLC peaks. (see Figure 2). Upon incubation with Tris-HCl at pH 8 for 20 h, 11% of the triester was hydrolyzed. At pH 9, a 20 h incubation resulted in 40% of the hydrolysis product. The triester was completely hydrolyzed within 15 h at pH 10 and within 1 h at pH 11 (see Figure 2). HPLC analysis confirmed that hydrolysis occurred by cleavage of the P-S bond and formation of TpT as expected.

To further characterize the reaction of monobromobimane with a phosphorothioate diester, the reaction was performed with an oligodeoxynucleotide which at ambient temperature exists largely in the double-stranded form. The reaction of the octamer d[GpCp(s)CpCpGpGpGpC] with a 10-fold excess of monobromobimane was performed in either distilled water or Tris-HCl pH 7, at ambient temperature. The HPLC analysis after a 5-h incubation (Figure 3) showed the starting material (14.88 min), a monobromobimane hydrolysis product (15.3 min), a product peak (17.75 min), and

monobromobimane (25.21 min). The starting material was completely consumed within 23 h. With a 5-fold excess of monobromobimane, the reaction was complete within 48 h. The reaction proceeded equally well with either the R_p or the S_p diastereoisomer. A control reaction containing an oligodeoxynucleotide with only phosphodiesters failed to show any conversion to a labeled product.

4) ^{31}P NMR Studies

The ^{31}P NMR studies were done at 121.5 MHz using a varian multinuclear FT-NMR. Positive chemical shift values are reported in parts per million (ppm) downfield from the external standard of aqueous 85% phosphoric acid. NMR analysis was done on a sample containing 1.2 umol of Tp(s)Tp(s)Tp(s)T (3.5 umol of phosphorothioate diesters) and 20 mM Na_2EDTA. The sample was adjusted to a volume of 250 uL with D_2O. After NMR analysis of the tetramer, 10 umol of monobromobimane (a 3-fold excess with respect to the diesters) in 100 uL of acetonitrile was added to the NMR tube with a final volume of 350 uL. The sample was allowed to react for 2.5 h at ambient temperature in the dark. NMR analysis was then repeated.

5) Radioisotopic Labeling (^{32}P End Labeling)

A reaction mixture in a final volume of 200 uL containing 40.1 uM 15-mer (1 A_{260} unit), 40.7 uM ATP, 10 mM $MgCl_2$, 10 mM dithiothreitol, 5 ug/mL bovine serum albumin, 40 mM Tris-HCl, pH 8.7, 0.127 uM (0.152 mCi) [γ-^{32}P]ATP, and 10 units of T_4 polynucleotide kinase was incubated at 37°C for 18 h. After the addition of the reaction mixture to the Sep-pak cartidge (prewashed with 20 mL of methanol and 20 mL of distilled water), it was washed with 10 mL of 1% aqueous methanol to elute the unincorporated ATP and buffer salts. The oligodeoxynucleotide was eluted with 10 mL of 50% aqueous

methanol. The solution containing the DNA fragment was evaporated to dryness and redissolved in 0.4 M distilled water. Isolated yields ranged from 60 to 80%.

The 21-mer, 23.3 uM (1 A_{260} unit), was end labeled in an analogous manner but could not be eluted with aqueous methanol. In this case, the Sep-pak cartidge was prewashed with acetonitrile and distilled water. The unincorporated ATP and salts were then eluted with 1% aqueous acetonitrile while the oligodeoxynucleotide was eluted with 50% aqueous acetonitrile. Isolated yields also ranged from 60 to 80%.

6) Post-Assay Labeling

Gel electrophoreseis was performed on 20 x 20 x 0.04 cm or 34 x 42 x 0.04 cm gels of 20% acrylamide, 2% bis(acrylamide) [or 6% acrylamide and 0.6% bis(acrylamide)], 50 mM Na_2EDTA, and 13 mM sodium persulfate. Post-assay labeling was performed both in the presence and in the absence of 7 M urea. The DNA was fixed in the gel by soaking it in 10% aqueous acetic acid for 5 min. The gel was then transferred to a 4 mM solution of monobromobimane in 50% aqueous acetonitrile and allowed to react overnight (18 h) in the dark. The gel was destained by shaking in 50% aqueous acetonitrile for 1 h. The short destaining appeared necessary because of minor reactions with the gel components and monobromobimane. Following a brief treatment (5 min) in 60 or 75% aqueous simethylformamide, the DNA was viewed on a standard long-wavelenth ultraviolet transilluminator (λmax = 366 nm). In some cases for internal standardization, the fluorescent bands of DNA were cut out of the gel and lyophilized before determination of the amount of DNA present in the gel via scintillation counting.

The effect of solvents on fluorescent intensity was also investigated. After post-assay labeling and destaining, the gels were treated with one of the following: 75% aqueous mixtures of methanol, ethanol, butanol, dimethylformamide, or concentrated glycerol. The gels were viewed using a long ultraviolet wavelength light transilluminator.

7) Fluorescent Studies

The fluorescense (excitation 385 nm, emission 465 nm) of varying solutions of bimane-labeled Tp(s)T in 5 mM KH_2PO_4, pH 4.5, was measured by using a fluorescence spectrophotometer, and a standard curve of fluorescence vs. phosphorothioate diester concentration was fitted to the data employing a linear least-squares analysis.

After post-assay fluorescent labeling (see above) with monobromobimane, the 5'-^{32}P end-labeled 15-mer was electroeluted for 2 h from a 20% polyacrylamide gel into dialysis tubing containing 0.5x TBE buffer. The solution was evaporated to dryness, redissolved in 1 mL of distilled water, and desalted using a column of Sephadex G-10. The DNA fragment was collected, evaporated to dryness, and redissolved in 3 mL of 5 mM KH_2PO_4, pH 4.5. The fluorescence of the solution was measured and the concentration of the 15-mer determined by scintillation counting. The fluorescence as a function of concentration of the phosphorothioate diesters was plotted on the standard bimane-labeled Tp(s)T curve.

In similar fashion, the 5'-^{32}P end-labeled 21-mer was electroeluted for 24 h from the polyacrylamide gel after post-assay labeling. The solution was evaporated to dryness and redissolved in 0.5 mL of distilled water. In this case, the solution containing the 21-mer was adjusted to 10 mM $MgCl_2$ and 2 M ammonium acetate, 1 volume of ice-cold

acetonitrile was added, and the solution was kept at -70°C for 18 h. The salt precipitated out of solution while essentially all of the DNA remained in the supernatant. The solubility of the labeled 21-mer in acetonitrile is largely a result of the increased hydrophobicity conferred upon the oligonucleotide due to the presence of the bimane residues. The supernatent was decanted, evaporated to dryness, and dissolved in 3 mL of 5 mM KH_2PO_4, pH 4.5. The fluorescence and radioactivity were measured and compared with the standard curve.

8) DNA Polymerase and Restriction Endonuclease Reactions

M13 mp18 DNA was converted to the replicative form (RF) as follows. The template DNA (2.5 ug) and universal primer (0.1 ug) were annealed in 25 uL of buffer containing 100 mM NaCl, 20 mM $MgCl_2$, and 100 mM Tris-HCl, pH 8.0, by heating the mixture to 56°C for 15 min followed by slow cooling to ambient temperature. The final 50-uL reaction mixture containing dATP, dGTP, dCTP, dTTP (500 uM each), ATP (1 mM), DNA polymerase 1 (Escherichia coli, 10 units), and T4 DNA ligase (8 units) was incubated overnight at 16°C. Substitution of the appropriate dNTPαS derivative(s) for the corresponding dNTP(s) essentially as described (Taylor, et al., Nucleic Acids Res., 13: 8749-8764, 1985) allowed the enzymatic incoporation of phosphorothioate diesters in place of phosphodiesters. In some cases for internal standardization, α-[^{35}S]-dATP (1.15 Ci/mmol) was employed in the elongation reaction.

Restriction digests with AvaI and HpaII were performed as follows. The AvaI reaction mixture contained RF M13mp19 DNA, 100 mM NaCl, 20 mM $MgCl_2$, and 100 mM Tris-HCl, pH 8.0. The HpaII reaction mixture contained RF M13mp18 DNA, 3 mM KCl, 5 mM $MgCl_2$, 100 ug/mL BSA, and 5 mM Tris-HCl,

pH 7.4. The reactions were initiated by the addition of the enzyme and incubated at 37°C for 2 h. The reaction mixture was loaded onto 6% acrylamide, 0.6% bis(acrylamide) gels (20 x 20 x 0.04 cm or 34 x 42 x 0.04 cm) containing 3 mM Na_2EDTA, 7 M urea, and 50 mM Tris-borate, pH 8.3. Fluorescent labeling proceeded as described above.

9) Detection of Nucleic Acids

The 5'-^{32}P end-labeled 21-mer was viewed on a transilluminator (λ_{max} = 366 nm) after gel analysis and post-assay labeling. The bluish green bands were excised from the gel and lyophilized, and the amount of DNA present was determined by scintillation counting. The amount of the oligodeoxynucleotide visible as a result of the bimane fluorescence has decreased such that 500 fmol (500 x 10^{-15} mol) of the DNA fragments could be observed.

Longer DNA fragments containing phosphorothioates can be prepared by enzymatic synthesis if the dNTP substrates are substituted by the α-thio derivatives (Taylor et al., Nucleic Acids Res., 13: 8749-8764, 1985). In order to generate fragments of defined length, an oligonucleotide primer was extended using an M13mp18 or M13mp19 template and the resulting material was hydrolyzed with a restriction endonuclease. It was possible to prepare M13 RF DNA containing phosphorothioates at each position. Cleavage of the elongated DNA with HpaII produced fragments which migrated in the 6% polyacrylamide gel and could be visualized by post-assay fluorescent labeling (Figure 4A). A similar experiment with the AvaI restriction endonuclease produced a 444-nucleotide fragment which could be visualized by post-assay covalent labeling (Figure 4B). Some high molecular weight DNA could also be observed in this gel at the edge of the sample well (Figure 4B). With the 444-mer,

the bands were excised, and the amount of DNA was determined by scintillation counting. Approximately 40 fmol (40×10^{-15} mol) of the 444-mer (containing a maximum of 104 bimane-labeled phosphorothioate diesters) could be visualized in this experiment.

10) Synthesis of oligodeoxynucleotides containing a single phosphorothioate diester

Two oligonucleotides were synthesized for covalent attachment of a variety of reporter groups, including spin labels, fluorophores and drug derivatives. A dodecadeoxynucleotide and an eicosodeoxynucleotide were chemically synthesized by the phosphoramidite method described in Example 1 and altering the oxidation step at the appropriate cycle, resulting in two phosphorus diastereomers (Rp and Sp). It is possible to prepare the oligonucleotide such that it contains a pure phosphorus diastereoisomer as described [Connolly et al., Biochemistry 23: 3443-3453, 1984; Taylor et al., 1985].

Specifically, the dodecamer has the sequence d[CGCA(s)AAAAAGCG] and the eicosomer has the sequence d[CGTACTAGTT(s)AACTAGTACG].

Additionally Tp(s)T was reacted with a number of fluorophores or reporter groups containing a variety of functional groups. Three functionalities, γ-bromo-α,β-unsaturated carbonyls, iodo (or bromo) acetamides, and aziridinyl sulfonamides, were observed to effectively label phosphorothioate diesters and produce the corresponding phosphorothioate triester carrying the desired reporter group.

11) Phosphorothioate triester oligodeoxynucleotides carrying various reporter groups

Oligodeoxynucleotides of Example 10 containing a single covalently bound reporter group (Fig. 5) were obtained by incubation of the phosphorothioate-containing DNA fragment with the reporter group of choice in aqueous or largely aqueous solutions at pH values from 5 to 8. These reactions were performed at 25 to 50°C and usually proceeded with yields greater than 85% after 24 h at 50°C. Resolution of the reaction mixture and isolation of the triester product was accomplished by using HPLC (4.6 X 250 mm Hypersil-ODS with 0.02 M KH_3PO_4 pH 5.5 and a methanol gradient). Modification of the phosphorothioate was observed to be more efficient for the single-stranded dodecamer than the self-complementary eicosomer. This difference in reactivity was partially overcome when the reaction mixture was heated at 50°C. In the absence of the phosphorothioate diester, control reactions using native oligodeoxynucleotides did not result in any significant labeling.

a) Attachment of a PROXYL spin label:

The reaction to produce the compound in Fig. 5a was conducted as described above using the following specific conditions: 10 mM 3-(2-iodoacetamido)PROXYL, 0.15 mM dodecamer, pH 8.0 (phosphate) at 50°C in a solution containing 4% DMP. Similar conditions were employed to label the eicosomer.

b) Attachment of a CC-1065 drug analogue: a derivative of the dihydropyrroloindole subunit:

The reaction to produce the compound in Fig. 5b was conducted as described above using the following specific conditions: 5 mM dihydropyrroloindole derivative,

0.07 mM dodecamer, pH 8.0 (Tris) at 50°C in a solution containing 60% DMF. This reaction required 48 h at 50°C or 80 h at 25°C at which time it was 70-80% complete. Similar conditions were employed to label the eicosomer.

c) Attachment of a sulfonamide-linked dansyl fluorophore:

The reaction to produce the compound in Fig. 5c was conducted as described above using the following specific conditions: 12 mM N-dansylaziridine, 0.34 mM dodecamer, pH 8.0 (phosphate) at 25°C in a solution containing 50% acetonitrile. Similar condiitons were employed to label the eicosomer.

At 50°C, HPLC analysis of the dansylaziridine reaction indicated the presence of minor products, suggesting some nonspecific reaction with the DNA. Labeling conducted at 25°C (pH 8.0) proceeded more slowly, but did not indicate the presence of any species other than the desired product and starting materials. However, the possibility of some nonspecific modification of the DNA even at 25°C can not be excluded.

d) Attachment of an N-linked dansyl fluorophore:

The reaction to produce the compound in Fig. 5d was conducted as described above using the following specific conditions: 10 mM 1,5-I-AEDANS, 0.80 mM dodecamer, pH 6.0 (phosphate) at 50°C in a solution containing 25% DMF. Similar conditions were employed to label the eicosomer.

12) Stability and properties of phosphorothioate triesters from examples 10 and 11

The unlabeled dodecamer helix, d[CGCA(s)AAAAAGCG] d[CGCTTTTTTGCG], exhibited a T_m of 55°C, and this was indistinguishable from the T_m values obtained for the PROXYL-labeled (a in Figure 5) or drug-labeled (b in Figure 5)

helices. The T_m value for the self-complementary eicosomer, d[CGTACTAGTT(s)AACTAGTACG]$_2$ with two labels was also largely unchanged (68.5°C) in comparison to the unlabeled fragment (T_m = 67°C).

The hydrolytic stability of the phosophorothioate triesters is an important practical consideration for the value of such derivatives in many studies. Hydrolysis of the triesters proceeded by desulfurization (monitored by HPLC and confirmed by comparison with authentic standards). No detectable cleavage of the oligodeoxynucleotide at the point of attachment was observed. This agrees with the results of ethylated or hydroxyethylated derivatives, which result in primarily desulfurization and only very minor amounts of chain cleavage.

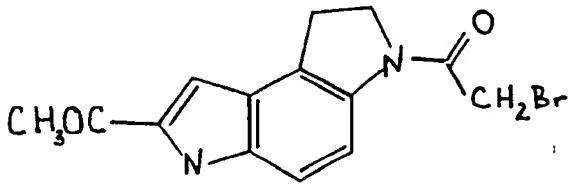
Less than 5% of the Tp(s)T triester carrying the PROXYL spin label was hydrolyzed after 24 h at pH 7. At pH 8 this increased to 28%, and at pH 10 the triester was completely hydrolyzed within 11 h. With longer fragments, the hydrolytic stability of the triester increased [the labeled dodecamer was hydrolyzed $<$1%, 30%, and 99% at pH values 7, 8, and 10, respectively; the values for the eicosomer were $<$1%, 2%, and 63%(24 h)]. The triester prepared from a γ-bromo-α,β-unsaturated carbonyl (b in Figure 5) exhibited stability similar to that of the PROXYL-labeled derivatives while that resulting from reaction with the aziridinyl sulfonamide (c in Figure 5) was more stable [the Tp(s)T-labeled triester was hydrolyzed $<$1% (pH 7), 5%(pH 8), and 34% (pH 10) after 24 h at ambient temperature].

It is noteworthy that the triester produced from 1,5-I-AEDANS and Tp(s)T was significantly less stable than the PROXYL-labeled derivative although the triesters formed

both resulted from iodoacetamides. The AEDANS-labeled dimer exhibited 19% (pH 7) and 88% (pH 8) hydrolysis (24 h); it was completely hydrolyzed within 2 h at pH 10. However, the AEDANS-labeled dodecamer (d in Figure 5) exhibited only 1%, 49%, and 99% hydrolysis at the same respective pH values (24 h).

An additional dodecamer was labeled with the bromoacetamideo derivative i. Although the three acetamido-linked adducts are similar in structure, that prepared from i proved to be more stable than either a or d (Figure 1) (only 13% of the triester formed from i was hydrolyzed after 24 h at pH 8.0).

Derivative i:



WE CLAIM:

1. A nucleic acid detection product comprising a phosphorothioate diester covalently complexed with an internucleotidic residue wherein said phosphorothioate diester is also complexed with a detectable marker.

2. The product of Claim 1 wherein said marker is a fluorescent marker.

3. The product of Claim 2 wherein said fluorescent marker is monobromobimane.

4. The product of Claim 2 wherein said fluorescent marker is bromomethylcoumarin.

5. The product of Claim 2 wherein said fluorescent marker carries a bromoacetamide, iodoacetamide, an aziridinosulfonamide, or a γ-bromo-α,β-unsaturated carbonyl group.

6. The product of Claim 1 wherein said marker is biotin, or a biotin derivative.

7. The product of Claim 1 wherein said marker is a spin label or spin probe.

8. The product of Claim 7 wherein said spin label is PROXYL.

9. The product of Claim 1 wherein said marker is a metal complex.

10. The product of Claim 1 wherein said marker is a drug or a drug analog.

11. The product of Claim 10 wherein said drug analog is a dihydropyrroloindole subunit of CC-1065.

12. A method of labeling nucleic acids comprising reacting a nucleic acid having a phosphorothioate diester in at least one internucleotidic phosphorus residue, with a marker to form an internucleotidic residue-phosphorothioate diester-marker complex.

13. A method of detecting nucleic acids, comprising introducing at least one phosphorothioate diester into a DNA fragment or oligodeoxynucleotide, labeling said phosphorothioate diester with a marker, and detecting the marker in complex with the nucleic acid.

14. A method of identifying nucleotides comprising site-selectively introducing at least one phosphorothioate diester into at least one internucleotidic residue of a DNA fragment or oligodeoxynucleotide, labeling said phosphorothioate diester with a detectable marker, and detecting said marker.

15. The method of Claim 12, 13 or 14 wherein said marker is a fluorescent marker.

16. The method of Claim 15 wherein said fluorescent marker is monobromobimane.

17. The method of Claim 15 wherein said fluorescent marker is bromomethylcoumarin.

18. The method of Claim 15 wherein said fluorescent marker carries a bromoacetamide, iodoacetamide, an aziridinosulfonamide, or a γ-bromo-α,β-unsaturated carbonyl group.

19. The method of Claim 12, 13 or 14 wherein said marker is biotin or a biotin derivative.

20. The method of Claim 12, 13 or 14 wherein said marker is a spin label or spin probe.

21. The method of Claim 20 wherein said spin label is PROXYL.

22. The method of Claim 13 or 14 wherein said marker is a metal complex.

23. The method of Claim 12 wherein said marker is a drug or a drug analog.

24. The method of Claim 13 or 14, wherein said labeling is conducted subsequent to resolution of the nucleic acid by performance of a biochemical assay.

25. The method of Claim 13 or 14, wherein said labeling is conducted prior to resolution of the nucleic acid by a biochemical assay.

26. The method of Claim 24 or 25, wherein said biochemical assay is gel electrophoresis.

27. The method of Claim 12, 13 or 14 wherein said phosphorothioate diester is selectively introduced into a DNA fragment or oligodeoxynucleotide at a specific nucleotidyl site.

28. The method of Claim 27 wherein said phosphorothioate diester is selectively introduced into said DNA fragment or said oligodeoxynucleotide by an oxidation reaction in the presence of elemental sulfur, CS_2 and lutidine; and subsequently by a hydrolysis reaction in the presence of a base.

29. The method of Claim 27 wherein said phosphorothioate diester is introduced into said DNA fragment or said oligodeoxynucleotide by chemical or enzymatic techniques.

30. A method of DNA sequencing comprising:

a. introducing at least one phosphorothioate diester into at least one selected site of a DNA fragment or oligodeoxynucleotide by enzymatic dideoxy sequencing procedures;

b. labeling each of said phosphorothioate diester with a marker; and

c. detecting said DNA sequence.

31. The method of Claim 30 which further comprises generating said DNA fragment or oligodeoxynucleotide from dNTPαS derivatives and at least one of dideoxy derivative (ddNTP).

32. The method of Claim 30, wherein said detection procedure is automated.

33. A method of DNA hybridization comprising:

a. generating DNA fragments or oligodeoxynucleotides of reproducible size by selective chemical means;

b. resolving said DNA fragments or oligodeoxynucleotides by a biochemical assay;

c. hybridizing said DNA fragments or oligodeoxynucleotides to a DNA hybridization probe having at least on internucleotidic phosphorothioate diester;

d. labeling said DNA hybridization probe with at least one detectable marker after said hybridization;

e. detecting at least one marker in complex with said hybridized probe.

34. A method of DNA hybridization comprising:

a. generating DNA fragments or oligodeoxynucleotides of reproducible size by selective chemical means;

b. resolving said DNA fragments or oligodeoxynucleotides by a biochemical assay;

c. labeling a DNA hybridization probe having at least one internucleotidic phosphorothiate diester with at least one detectable marker before hybridization with said DNA fragments or oligodeoxynucleotides;

d. hybridizing said DNA fragments or oligodeoxynucleotides to said labeled DNA hybridization probe;

e. detecting at least one marker in complex with said hybridized probe.

35. The method of Claim 33 or 34 wherein said DNA hybridization probe has an internucleotidic phosphorothioate diester at each internucleotidic phosphorus.

36. The method of Claim 33 or 34 wherein said selective chemical means is digestion with restriction endonucleases.

37. The method of Claim 33 or 34 wherein said biochemical assay for resolution of said DNA fragments or oligodeoxynucleotides is polyacrylamide or agarose gel electrophoresis.

38. The method of Claim 33 or 34 wherein said DNA hybridization probe is prepared by the steps comprising:

a. obtaining DNA fragments from restriction digests; and

b. incorporating more than one phosphorothioate diester into said DNA fragments by DNA polymerase and nick translation procedures.

39. The method of Claim 33 or 34 wherein said DNA hybridization probe is prepared from mRNA.

40. The method of Claim 39 wherein said hybridization probe is prepared from mRNA by the steps comprising:

a. providing dNTPαS derivatives to function as substrates for reverse transcriptase; and

b. synthesizing a complementary DNA strand to said mRNA by the action of reverse transcriptose, thereby forming said hybridization DNA probe.

41. The method of DNA hybridization according to Claim 33 or 34 wherein said marker is a fluorescent marker.

42. The method of DNA hybridization according to Claim 33 or 34 wherein said marker is monobromobimane.

43. The method of DNA hybridization according to Claim 33 or 34 wherein said marker is bromomethylcoumarin.

44. The method of DNA hybridization according to Claim 33 or 34 wherein said marker carries a bromoacetamide, iodoacetamide, aziridinosulfonamide or γ-bromo-α,β-unsaturated carbonyl group.

45. The method of DNA hybridization according to Claim 33 or 34 wherein said marker is biotin or a biotin derivative, and wherein the resulting product is a biotin-labeled hybridization probe.

46. The method of Claim 45 wherein said detection comprises:

a. attaching a detectable protein to said biotin-labeled hybridization probe; and

b. identifying said probe.

47. The method of DNA hybridization according to Claim 46 wherein said protein is avidin or streptavidin.

48. The method of DNA hybridization according to Claim 46 wherein said protein is an antibody to said biotin-labeled hybridization probe.

49. The method according to Claim 33 or 34 wherein said DNA fragment or oligodeoxynucleotide is transferred to a blotting membrane for the detection of specific DNA sequences by Southern blots, Northern blots, colony screening or plaque screening, thereby identifying DNA sequences under investigation.

50. A method for DNA detection comprising:

a. generating a DNA probe from DNA fragments or oligodeoxynucleotides having at least one phosphorothioate diester;

b. hybridizing said DNA probe to a selected DNA sequence under investigation;

c. labeling said probe with at least one detectable marker subsequent to said hybridization and thereby introducing said marker into the phosphorothioate diester; and

d. locating said selected DNA sequences under investigation by detecting said marker complexed with said hybridization probe.

51. A method for identifying DNA sequences comprising:

a. generating a DNA probe from DNA fragments or oligodeoxynucleotides having at least one phosphorothioate diester;

b. hybridizing said DNA probe to a selected DNA sequence under investigation;

c. labeling said probe with at least one detectable marker subsequent to said hybridization and thereby introducing said marker into the phosphorothioate diester;

d. locating said selected DNA sequences under investigation by detecting said marker complexed with said hybridization probe; and

e. directing drug delivery to said DNA sequence.

52. The method of Claim 51 which further comprises activating said DNA sequence.

53. The method of Claim 51 which further comprises inactivating said DNA sequence.

54. The method of Claim 51 which further comprises degrading said DNA sequence.

55. A method of targeting a nucleic acid for sequence-specific drug delivery which comprises:

a. preparing a sequence-specific nucleic acid probe having at least one phosphorothioate diester;

b. labeling said phosphorothioate diester of said probe with a drug or a drug analogue; and

c. hybridizing said probe with said nucleic acid and thereby delivering said drug to a specific nucleic acid target.

56. The method of Claim 55 wherein step b is performed subsequent to step c.

57. The method of Claim 55 wherein said drug analogue is a dihydropyrroloindole subunit of CC-1065.

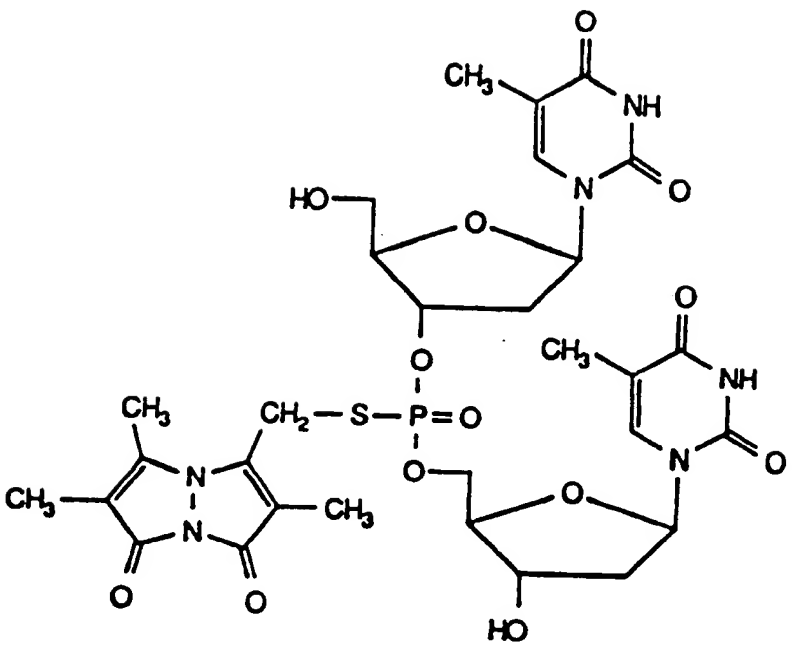


FIG. 1

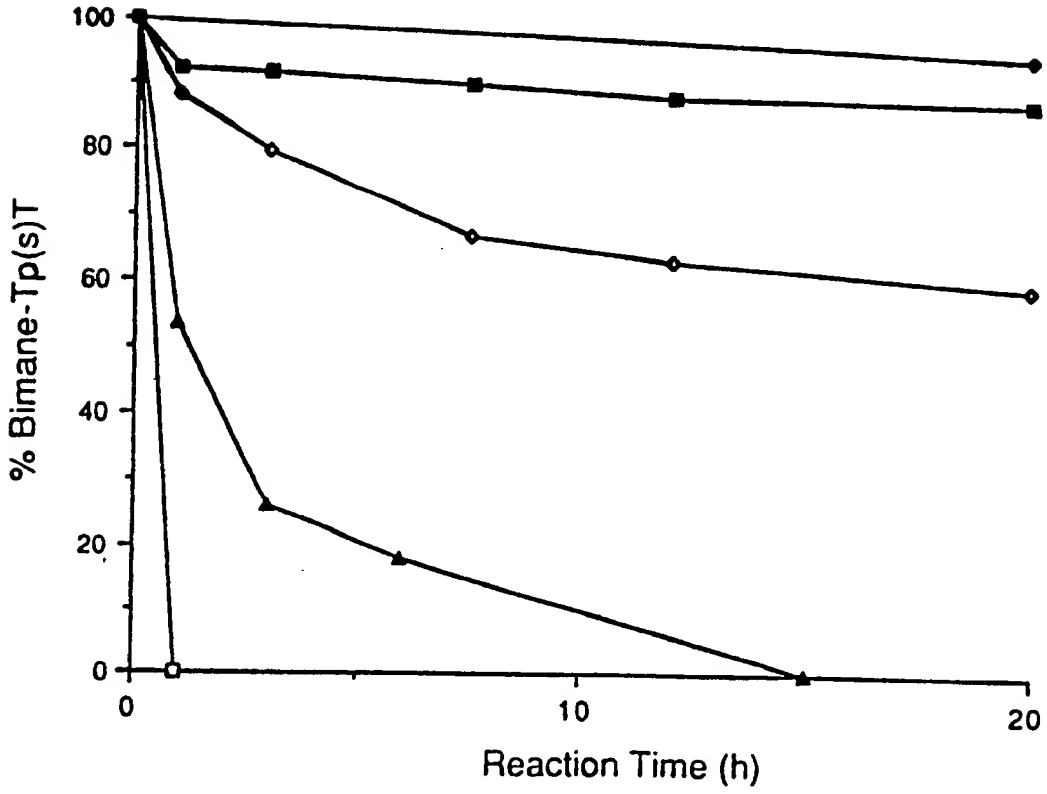


FIG. 2

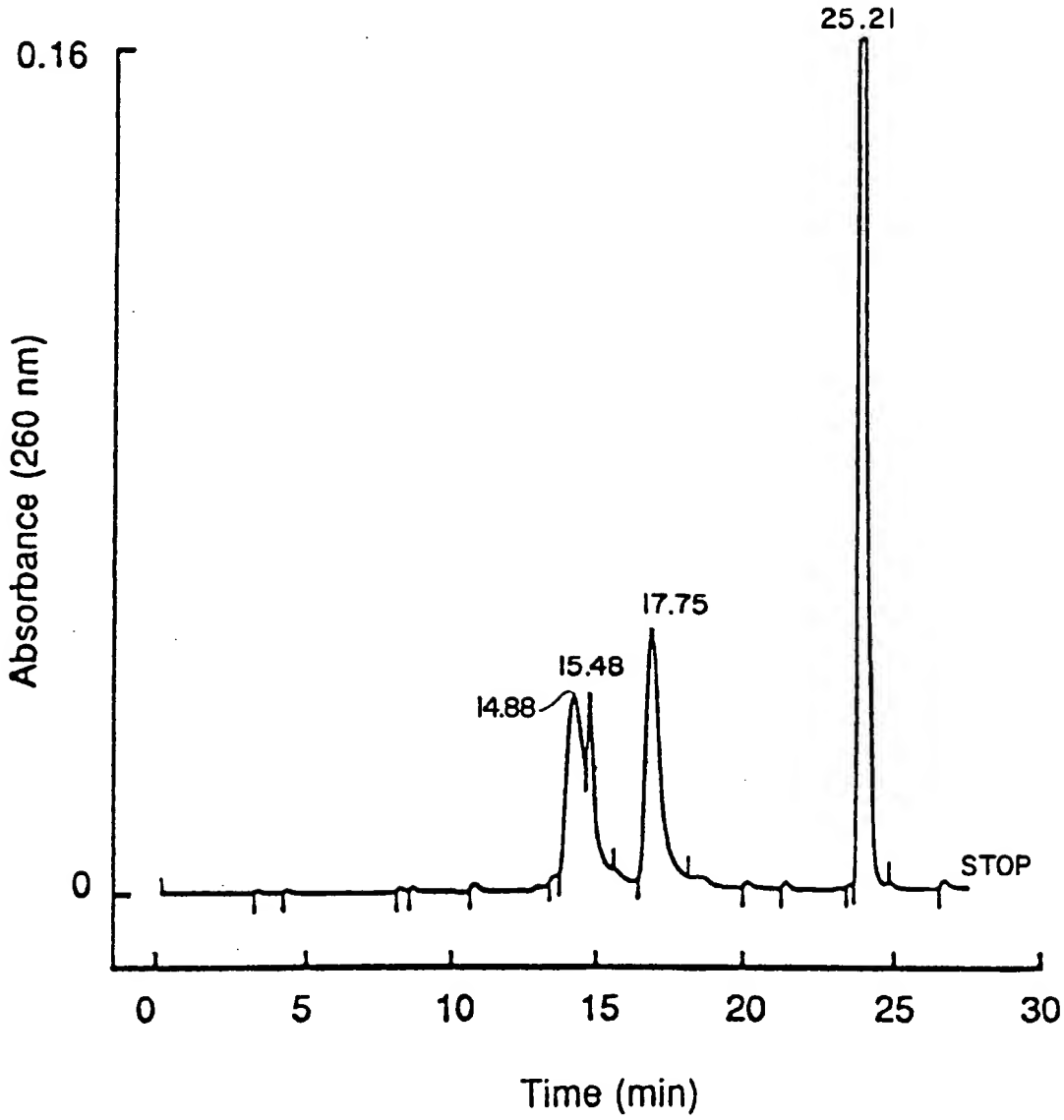

25.21
0.16
Absorbance (260 nm)
17.75
15.48
14.88
STOP
0
0
5
10
15
20
25
30
Time (min)

FIG. 3

FIG.4A

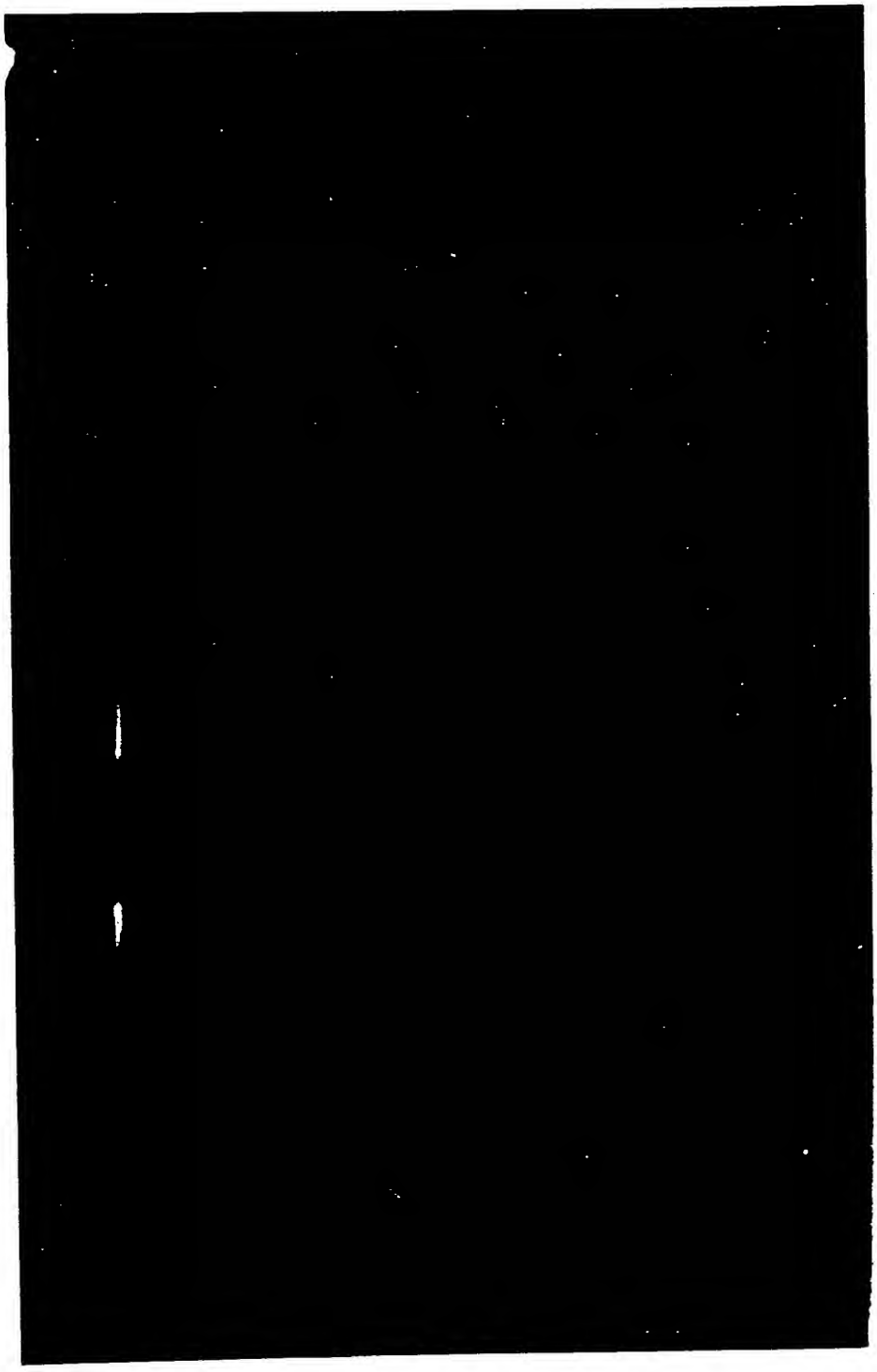


FIG.4B

FIG. 5b

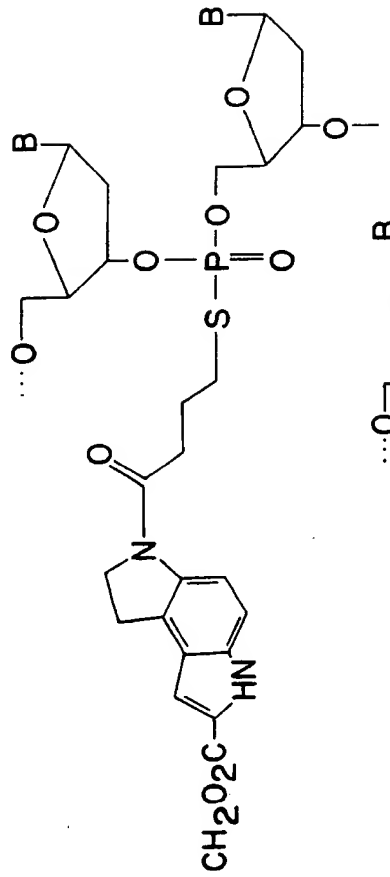


FIG. 5d

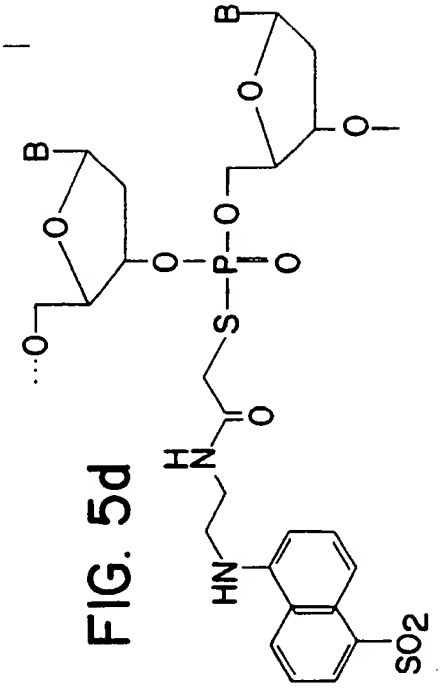


FIG. 5a

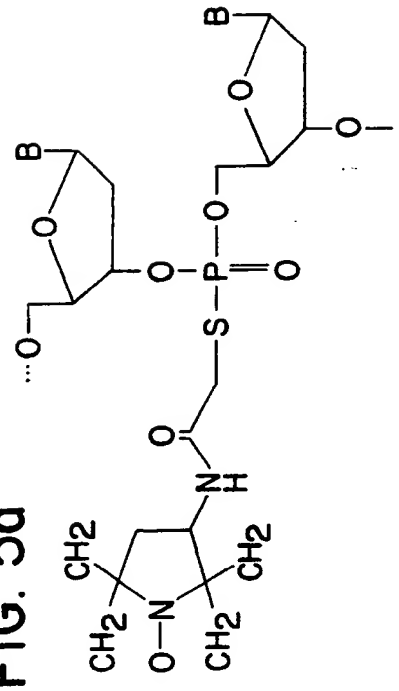
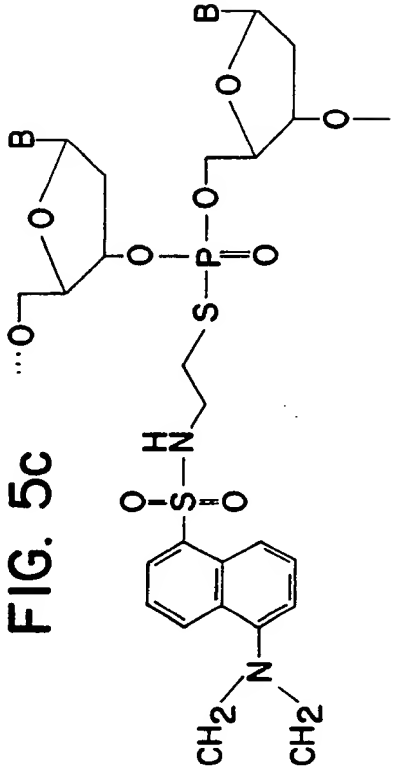


FIG. 5c



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00182

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/68

U.S. CL.: 435/6; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. CL.	435/6, 436/800, 803, 804, 536/27, 935/86

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Dialog Data Base: Biotech, APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,358,535, (FALKOW ET AL.) 09 November 1982 (See columns 4 and 5).	1-29 & 50
Y,E	US, A, 4,910,300, (URDEA ET AL.) 20 March 1990 (See examples 10-13 and the claims).	1-29 & 50

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02 MAY 1990	11 JUN 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	AMELIA BURGESS YARBROUGH

Form PCT/ISA/210 (second sheet) (Rev.11-87)